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14. ABSTRACT

BRCA1, the protein product of the Breast Cancer Susceptibility Gene (BRCA1) has been implicated in multiple pathways that preserve genome stability, including cell cycle control, DNA repair, ranscription, and chromatin remodeling. BRCA1, in complex with another RING-domain protein BARD1, possesses ubiquitin-ligase activity. Only a few targets for this activity have been identified in vivo. Nucleosomal histones may be among these targets since they can be modified by BRCA1/BARD1 in vitro. Here we demonstrate that the BRCA1/BARD1 complex can ubiquitylate both free H2A and H2B histones and histones in the context of nucleosomal particles. We have also investigated the possibility that BRCA1/BARD1 can attach ubiquitin to H2A and H2B residing on the same particle. These results raise the possibility that BRCA1/BARD1 can directly affect nucleosomal structure, dynamics, and function through its ability to modify nucleosomal histones.

15. SUBJECT TERMS
BRCA1, Ubiquitylation, Histone H2A, Histone H2B, Nucleosome

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Introduction

Mutational inactivation of BRCA1 accounts for 50-90% of hereditary breast and ovarian cancers. BRCA1 has been implicated in multiple pathways that preserve genome stability, including cell cycle control, DNA repair, transcription, and chromatin remodeling (Scully and Livingston, 2000; Starita and Parvin, 2003; Lane, 2004; Mullan et al., 2006). The multifunctional nature of BRCA1 has raised the possibility that the protein may employ a common mechanism, such as reorganizing chromatin structure, to regulate various chromosomal events. Indeed, BRCA1 is associated with histone modifying enzymes (p300 and HDAC) (Yarden and Brody, 1999; Pao et al., 2000) and ATP-dependent chromatin remodeling machines (hSWI/SNF) (Bochar et al., 2000). Moreover, targeting of BRCA1 to a heterochromatic chromatin region results in large-scale chromatin decondensation, which is not accompanied by histone hyperacetylation (Ye et al., 2001). Many cancer-predisposing mutations reduce BRCA1's affinity for these chromatin-modifying proteins, suggesting that chromatin remodeling may be an important aspect of BRCA1 tumor-suppression activity. BRCA1 can also function via an alternative mechanism, by directly affecting nucleosome structure and dynamics through its proven protein ubiquitylation activity. Indeed, the RING finger of BRCA1 confers ubiquitin ligase activity to the molecule, and this activity is markedly enhanced when BCRA1 forms a complex with another RING protein BARD1 (Hashizume et al., 2001; Starita and Parvin, 2006; Boulton, 2006; Barber and Boulton, 2006). The BRCA1/BARD1 complex mono-ubiquitylates purified core histones *in vitro* (Chen et al., 2002; Mallery et al., 2002). Whether core histones can be modified in the context of chromatin is unclear.

Despite the fact that histones (H2A) were the first proteins described to be ubiquitylated (Goldknopf et al., 1975; Goldknopf and Busch, 1977), the structural and functional consequences of this bulky post-translational modification remain unclear. The histone ubiquitylation field has experienced a burst of renewed interest following the identification of the E2 ubiquitin-conjugating and E3 ubiquitin-ligating enzymes that covalently attach ubiquitin to histone H2B lysine 123 in yeast (Robzyk et al., 2000; Hwang et al., 2003; Wood et al., 2003). The yeast studies were quickly followed by identification of the mammalian enzymes that modify H2BK120 (Zhu et al., 2005; Kim et al., 2005) or H2AK119, a modification that is not present in yeast (Wang et al., 2004; Cao et al., 2005). Equally importantly, several laboratories have described a unidirectional, evolutionarily-conserved "trans-tail" event, in which H2Bub regulates subsequent methylation of histone H3 at K4 and K79 (Dover et al., 2002, Ng et al., 2002, Sun and Allis, 2002; Briggs et al., 2002; Kim et al., 2005; Pavri et al., 2006), suggesting a role in transcriptional regulation (for reviews and further references, see Osley, 2006a, b; Laribee et al., 2007). The importance of this trans-tail connection for transcription regulation has been recently challenged by at least one *in vitro* (Pavri et al., 2006) and several *in vivo* studies (Shukla and Bhaumik, 2007;Tanny et al., 2007), all pointing to a predominant transcriptional role for H2B ubiquitylation which is independent of H3 methylation.

Relevant to the present study is also the notion that H2A and H2B ubiquitylation play distinct, and in many cases opposing roles in transcriptional regulation. H2Bub occurs

throughout the promoter and coding regions and participates in both gene activation and in gene silencing (Osley, 2006a, b; Laribee et al., 2007). H2Aub, on the other hand, is strictly confined to the promoter regions and is involved in transcriptional silencing (Osley, 2006a, b). How this specificity in regulation of transcription is achieved is unclear. In theory, two major, mutually non-exclusive mechanisms are possible: specific interactions of ubiquitylated H2A and H2B with protein factors and direct differential effects of ubiquitylated H2A and H2B on the structural and dynamic properties of the nucleosome or the chromatin fiber.

As a first step in elucidating the properties of nucleosomes that contain either H2Aub or H2Bub, we setup an *in vitro* ubiquitylation system using the BRCA1/BARD1 complex (E3). BRCA1/BARD1, in combination with UbcH5c or UbcH5a E2 enzymes, has been reported to ubiquitylate purified histones (Chen et al., 2002; Mallery et al., 2002). However, whether it can modify nucleosomal histones has not been investigated. Here we show: (i) that both H2A and H2B can be ubiquitylated when organized in the nucleosome; and (ii) in the absence of additional factors, nucleosomes can be simultaneously modified on both histones.

Body

Task 1: Determine the histone ubiquitylation activity of BRCA1 on histones incorporated into reconstituted nucleosome particles (months 1-4).

a. Acquire existing expression clones for human BRCA1 and BARD1 proteins, and purify the recombinant proteins (months 1-2). Acquired BRCA1/ BARD1 expression clones from Dr. Zhen-Qiang Pan (Derald H. Ruttenberg Cancer Center, The Mount Sinai School of Medicine, New York, New York 10029-6574, overexpressed and purified the proteins. Unfortunately they were not active. Hence we collaborated with Dr. Jaffery Parvin (Department of Biomedical Informatics, The Ohio State University Medical Center, Columbus, OH 43210), who provided BRCA1/BARD1 protein complex.

b. Acquire and perform quality tests on the other components of the ubiquitylation system: E1 and UbcH5a (Boston Biochem), ubiquitin (Sigma), and individual core histones (Roche) (months 1-2). Done

c. Form the BRCA1/BARD1 complex and test its in vitro ubiquitylation activity on purified histones (month 3). Done

d. Reconstitute nucleosome particles on the 208 bp nucleosome positioning sequence from sea urchin 5s rRNA gene; perform and analyze the ubiquitylation reaction (month 4). Done

Task 2: Use single-molecule assays to study the effect of BRCA1-mediated ubiquitylation of core histones on the dynamics of ‘canonical’ nucleosomal particles in real time (months 5-12). To be performed

a. Perform all labeling procedures to attach the pair of fluorescent dyes (Cy3 donor and Cy5 acceptor) to specific sites in the nucleosome particle (either both dyes at different positions of the DNA to follow dissociation/opening or breathing) or to the DNA and one of the histones to follow sliding) (month 5).
b. Using the existing wide-field prism-based evanescent field fluorescence microscope (EFFM) perform the opening/breathing/sliding experiments to follow the spontaneous conformational transitions in control nucleosome particles containing unmodified histones (months 6-9).
c. Repeat the above EFFM experiments on nucleosome containing the BRCA1-ubiquitylated histones (months 10-12). The ubiquitylation reaction will be performed in three alternative ways:

c.1. On isolated histones, after which the ubiquitylated histones species will be reconstituted into nucleosomes (bulk reconstitution);
c.2. On pre-reconstituted nucleosome particles (bulk reconstitution), see Task 1;
c.3. On individual pre-reconstituted nucleosome particles attached to the glass surface in the flow cell of the instrument; in this specific case, one hopes to be able to see the conformational changes that occur in nucleosomes as a result of BRCA1-mediated modification in real time.

EXPERIMENTAL PROCEDURES

DNA Fragments for Nucleosome Reconstitution—Mononucleosome particles were reconstituted using the 208 bp nucleosome positioning sequence from sea urchin 5S rDNA, and oligonucleosomes were reconstituted on the same sequence repeated in tandem (208-12) (Simpson et al., 1985).

Isolation of Human Recombinant Histones H2A and H2B—The coding sequences for the canonical human histones H2A, H2B, H3, and H4 have been cloned using the pET-22b expression vector in Dr. Fukui's laboratory. Recombinant histones were then overexpressed in Rosetta (DE3) pLysS cells, purified by tandem ion exchange chromatography (Q Sepharose/SP Sepharose, Amersham Biosciences), and checked on 15% SDS-PAGE (**Fig. 1A**).

Preparation of the BRCA1/BARD1 complex— The BRCA1/BARD1 complex was purified from Hi-five cells co-infected with recombinant baculoviruses for the expression of BRCA1 and BARD1 and purified as described (Starita et al., 2004). The concentration of the complex was in the range of 50-100 nM.

Reconstitution of Histone Octamers and of Nucleosomes—Octamers were reconstituted by dialysis of an equimolar mixture of all four core histones from 8M guanidinium-HCl to 2M NaCl (Luger et al., 1999), followed by purification on a Superdex (Amersham Biosciences) column. Nucleosomes were reconstituted by the salt-jump method (Zivanovic et al., 1990) as detailed in Tomschik et al. (2005). Reconstitutes were analyzed on 5.5% native PAGE gels that resolve alternative octamer positions along the DNA sequence (**Fig. 2**). Oligonucleosomes were reconstituted by the same method using the 208-12 fragment and analyzed on 1% agarose gels (**Fig. 2**). We used conditions (histone/DNA ratios) which led to only partial assembly: the reconstitute contained less than half the number of possible nucleosomes, as judged by restriction nuclease digestion (Hansen et al., 1989). This condition was necessary to ensure that

neighboring modified particles will not interact with each other to cause aggregation (see below).

In vitro Ubiquitylation Assay and Analysis of the Modified Substrates—The ubiquitylation reaction mixture (30 µl) contained 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM ATP, 0.6 mM dithiothreitol, 3 µg ubiquitin, 1 µg of histones, 300 ng of E1 (Sigma), 300 ng of UbcH5c (Upstate) (Chen et al. 2002), and 40ng of the BRCA1/BARD1 complex (Starita et al., 2004). The reactions were performed at 37°C for 1.5 hrs and were stopped by adding 15 µl of 5x Laemmli loading buffer; the samples containing purified histones were boiled for 3 min, whereas the samples containing nucleosomes and oligonucleosomes were heated from 60°C to boiling for ~30 min prior to SDS-PAGE analysis. Proteins were resolved on 15% SDS-PAGE and further analyzed by Western blotting. Resolved proteins where transferred onto a nitrocellulose membrane (Bio-Rad) by electroblotting. Membranes were probed with one of the following antibodies, diluted 1:1000: anti-H2A C-terminus antibody (Abcam #18255, raised vs. H2A residues 115-129), anti-H2A acidic-patch antibody (Upstate # 07-146, raised vs. H2A residues 88-97), anti-H2Aub antibody (Millipore # 05-678 raised vs. ubiquityl-histone H2A) anti-H2B C-terminus antibody (Abcam #1790, raised vs. H2B residues 108-125), and anti-Ub antibody (Upstate #07-375). Alternatively, the blots were stripped by Restore™ Western Blot Stripping Buffer (Thermo Scientific) following the first Ab and further probed with another antibody. After incubation with a primary antibody for 2 hrs, the membranes were washed trice with water and then incubated for 30 min at room temperature with the secondary antibody (goat anti-rabbit HRP-conjugated IgG, Santa Cruz Biotechnology, SC-2004) diluted 1:10 000. The membrane was developed using the ECL Western blotting detection reagents according to manufacturer's protocol (Amersham Biosciences) and chemiluminescence was detected on autoradiography film.

RESULTS AND DISCUSSION

Both Histones H2A and H2B can be Modified when Free in Solution—Modification of purified histones free in solution by BRCA1/BARD1 has been previously reported (Chen et al., 2002; Mallery et al., 2002). The Chen et al. (2002) work focused on recombinant H2A from human origin, whereas the Mallery et al. (2002) paper demonstrated ubiquitylation of all core histones, but the origin of the histones was not specified. Thus, we performed ubiquitylation reactions on human recombinant histones. The quality of the purified proteins used for these reactions, and also for reconstituting nucleosomes and nucleosomal arrays, was checked by SDS-electrophoresis (**Fig. 1A**).

We next investigated the ubiquitylation of H2A by Western blot analysis. Transferred proteins were first probed with anti-H2A antibody, followed by membrane stripping and reprobing with anti-ubiquitin antibody to detect both ubiquitin and ubiquitylated proteins. The data (**Fig. 1B**) clearly indicate an efficient ubiquitylation of H2A, with at least 50% of the protein being modified (mono- and di-ubiquitylation). The specificity of the reaction was tested by performing the reaction in the absence of ubiquitin, the E1 enzyme, or BRCA1/BARD1 (**Fig. 1C**). Only in the presence of all components of the ubiquitylation machinery (E1, E2, BRCA1/BARD1, ubiquitin, and ATP, designated full reaction) was H2A modified.

We then performed a similar test on purified H2B. We again observed two modified forms (mono and di) but the reaction was much less efficient (**Fig. 1D**).

Thus, our *in vitro* ubiquitylation system modifies both histones H2A and H2B, H2A being modified with higher efficiency.

Both Histones H2A and H2B can be Modified when Incorporated in Mononucleosomes and Nucleosomal Arrays—Since our overall goal is to understand the mechanism of histone ubiquitylation action in the context of chromatin, we next performed the ubiquitylation reaction on mononucleosomes, reconstituted *in vitro* from a DNA fragment containing a nucleosome positioning sequence and purified recombinant human histones. The quality of reconstitution was checked on native polyacrylamide gels, in which the population of reconstituted particles separates into several bands that differ in the position of the histone octamer with respect to the underlying sequence (O'Donahue et al., 1994; Furrer et al., 1995) (**Fig. 2A**). The uppermost band corresponds to a particle in which the octamer occupies a central position on the DNA fragment, whereas the lowest band is an end-positioned nucleosome.

The Western blot was successively treated with anti-H2A antibody and then with anti-H2B antibody (**Fig. 2B**). It was clear that histone H2B has been modified, and the extent of modification was similar to that of the purified protein. The situation with nucleosomal H2A was much more difficult to interpret. Although we could see no band corresponding to modified forms of H2A, there was an almost complete disappearance of the unmodified band in the full reaction lane. This result was highly reproducible in experiments performed on mononucleosomal particles and in experiments performed with subsaturated nucleosomal arrays (**Fig. 2C** and **D**).

There were two possible explanations for this rather unexpected result. First, the anti-H2A antibody used recognizes the C-terminus of H2A, which contains the ubiquitylatable K120 residue. Thus, it is possible that the modified forms of H2A were no longer recognizable by this antibody. In order to exclude this possibility, we acquired a new anti-H2A antibody which was raised against peptide 88-99, i.e. should recognize the molecule independently of whether it is ubiquitylated or not. Using this antibody, we observed exactly the same behavior: disappearance of the H2A unmodified band form the gel (lanes 1 and 2 in the anti-H2A blot in **Fig. 2E**), and lack of appearance of new bands entering the gel.

The second possible explanation is that the ubiquitylated nucleosomal particles or nucleosomal arrays are prone to aggregation, and the aggregate does not enter the gel. Indeed, in later experiments, we carefully inspected the gels, the blots, and the Western blots for the presence of aggregated material in the wells. As seen in **Fig. 2E** (left panel), some aggregation occurred even during ubiquitylation of the purified H2A. The aggregation was much more pronounced in the ubiquitylated nucleosome preparation (seen in the blot and anti-Ub western blot, right panel of **Fig. 2E**). Again, though, the material in the well did not stain with anti-H2A antibody, most probably as a result of occlusion of the respective epitope in the aggregate.

Thus, we can conclude that both nucleosomal histones H2A and H2B are modified by BRCA1/BARD1. The degree of modification, however, is significantly different. H2A is massively (almost quantitatively) modified, whereas the ubiquitylation of H2B is relatively modest.

Both Histones H2A and H2B can be Modified on the Same Nucleosomal Particle— An important unresolved question concerning histone ubiquitylation in the context of the nucleosomal particle is whether or not a single particle can contain both H2Aub and H2Bub. Early biochemical experiments from J. Davie's laboratory (Li et al., 1993) used cross-linking of hydroxyapatite column-fractions that contained H2A, H2Aub, H2B, and H2Bub (but no DNA) and two-dimensional electrophoretic analysis of the cross-linked products. They observed that H2Aub and H2Bub could be crosslinked, indicating that H2Aub and H2Bub can form dimers in solution. With the advent of more sophisticated experimental techniques, it is now possible to ask the next question – can nucleosome particles accommodate both H2Aub and H2Bub in the same particle. The expectation from a structural view point is that this may be possible (**Fig. 3**). On the other hand though, the simultaneous presence of the two modified histones may be incompatible with their opposite role in transcription regulation (see Introduction).

In order to see whether BRCA1/BARD1 can modify both H2A and H2B in the same particle, we took advantage the availability of a commercial antibody that specifically recognizes monoubiquitylated H2A. (**The antibody is on backorder and will be available to us only in April)(experiments in progress**). We will use this antibody for immuno-selection of nucleosomes containing H2Aub; these nucleosomes will be then probed for the presence of modified H2B.

Key Research Accomplishments

We have demonstrated that both nucleosomal histones H2A and H2B are modified by BRCA1/BARD1. The degree of modification, however, is significantly different. H2A is massively (almost quantitatively) modified, whereas the ubiquitylation of H2B is relatively modest.

Reportable Outcomes

Manuscript in preparation, Abstract for the Era of Hope conference.

Conclusion

We conclude that both nucleosomal histones H2A and H2B are modified by BRCA1/BARD1. The degree of modification, however, is significantly different. H2A is massively (almost quantitatively) modified, whereas the ubiquitylation of H2B is relatively modest. These results raise the possibility that BRCA1/BARD1 can directly affect nucleosomal structure, dynamics, and function through its ability to modify nucleosomal histones.

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Appendices

SUPPORTING DATA

FIGURE LEGENDS

FIG. 1. **Purification of recombinant human core histones and *in vitro* ubiquitylation reaction performed with H2A and H2B.** *A*, Purified recombinant human histones are analyzed on Coomassie blue-stained 15% SDS-PAGE. *B*, BRCA1/BARD1 ubiquitylation of H2A: the nitrocellulose membranes were probed with the anti-H2A antibody, stripped, and re-probed with the anti-Ub antibody. *C,* BRCA1/BARD1-mediated ubiquitylation of H2A in a complete reaction mixture and in partial mixtures lacking ubiquitin, E1, or BRCA1/BARD1. Upper panel, Ponceau staining of the nitrocellulose membrane after electroblotting; lower panel, same membrane probed with anti-H2A antibody. *D*, BRCA1/BARD1 ubiquitylation of H2B: the nitrocellulose membranes were probed with the anti-H2B antibody, stripped, and re-probed with the anti-Ub antibody.

FIG. 2. **Reconstitution of mononucleosomes and oligonucleosomes and *in vitro* ubiquitylation reaction by BRCA1/BARD1.** *A*, Mononucleosomes reconstituted *in vitro* from the 208 bp DNA fragment and purified human histones and analyzed on SYBR Green I-stained 5.5% native PAGE. M, marker; R, reconstituted mononucleosome. *B*, BRCA1/BARD1 ubiquitylation of mononucleosomes: the nitrocellulose membrane was probed with the anti-H2A C-terminus antibody, stripped, and re-probed with the anti-H2B antibody. *C*, Oligonucleosomes reconstituted *in vitro* from the 208-12 DNA fragment and purified human histones and analyzed on ethidium bromide-stained 1% agarose gel. *D*, BRCA1/BARD1 ubiquitylation of oligonucleosomes: the nitrocellulose membrane was probed with the anti-H2A C-terminus antibody, stripped, and re-probed with the anti-H2B antibody. *E*, BRCA1/BARD1 ubiquitylation of H2A and mononucleosomes: the nitrocellulose

membrane was stained with Ponceau (labeled blot), washed and probed with the anti-H2A acidic-patch antibody, stripped, and re-probed with the anti-UB antibody. Dotted line boxes indicates the presence of material in full reaction sample wells when blot was stained by Ponceau and probed by anti-UB antibody, whereas the full line box indicates the loss of H2A in full reaction when probed with anti-H2A acidic-patch antibody.

FIG. 3. **Schematic representation of ubiquitin binding to nucleosomal H2A and H2B**. Representation of a portion of the crystal structure of the nucleosome core particle containing, for clarity, only the nucleosomal DNA and the two molecules of either H2B (panel *A*) or H2A (panel *B*). Panel *C* depicts a hypothetical particle with both H2A and H2B ubiquitylated. For clarity, only one H2A and one H2B molecule are shown. DNA double strands are shown in blue and yellow, histones are shown in red, and the ubiquitin molecule is shown in purple. The schematic was created from existing crystal structure coordinates from NCBI's PDB (2CV5) structure of the human nucleosome and PDB (2ZCB) of ubiquitin with the help of UCSF Chimera.

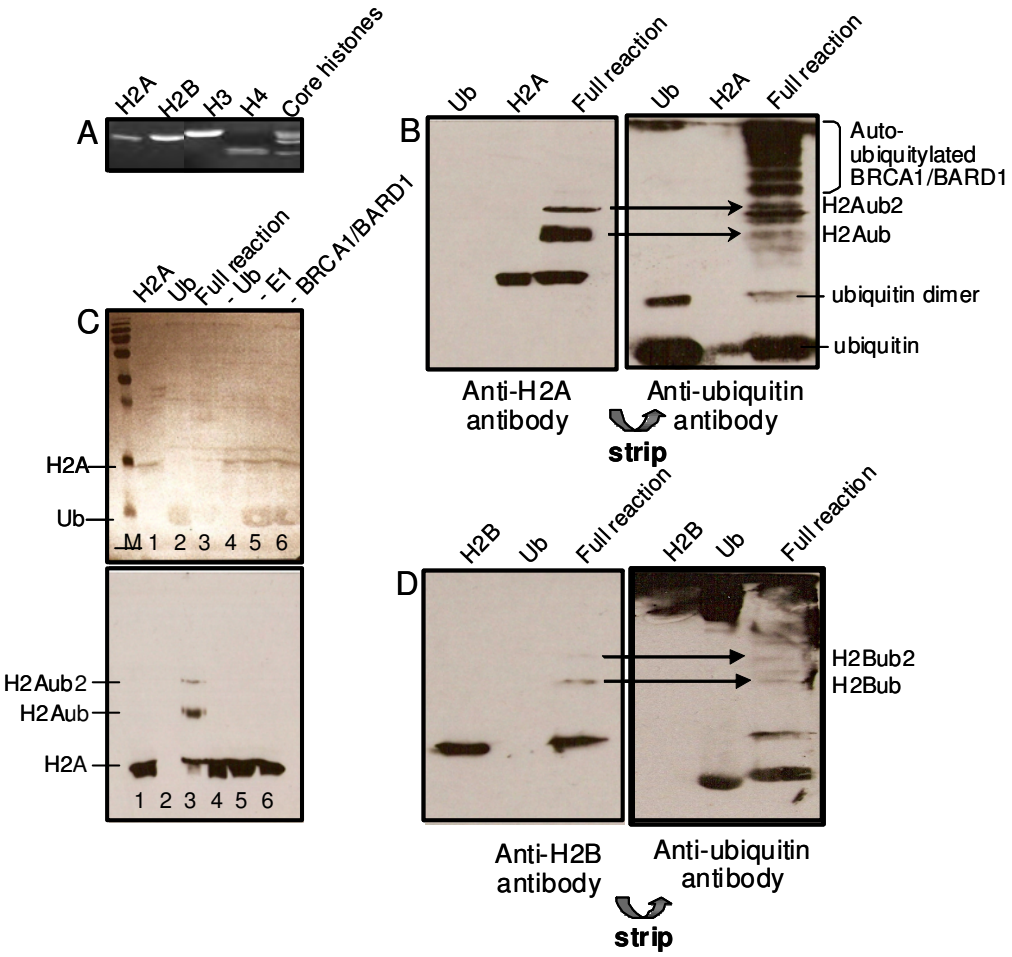

Figure 1
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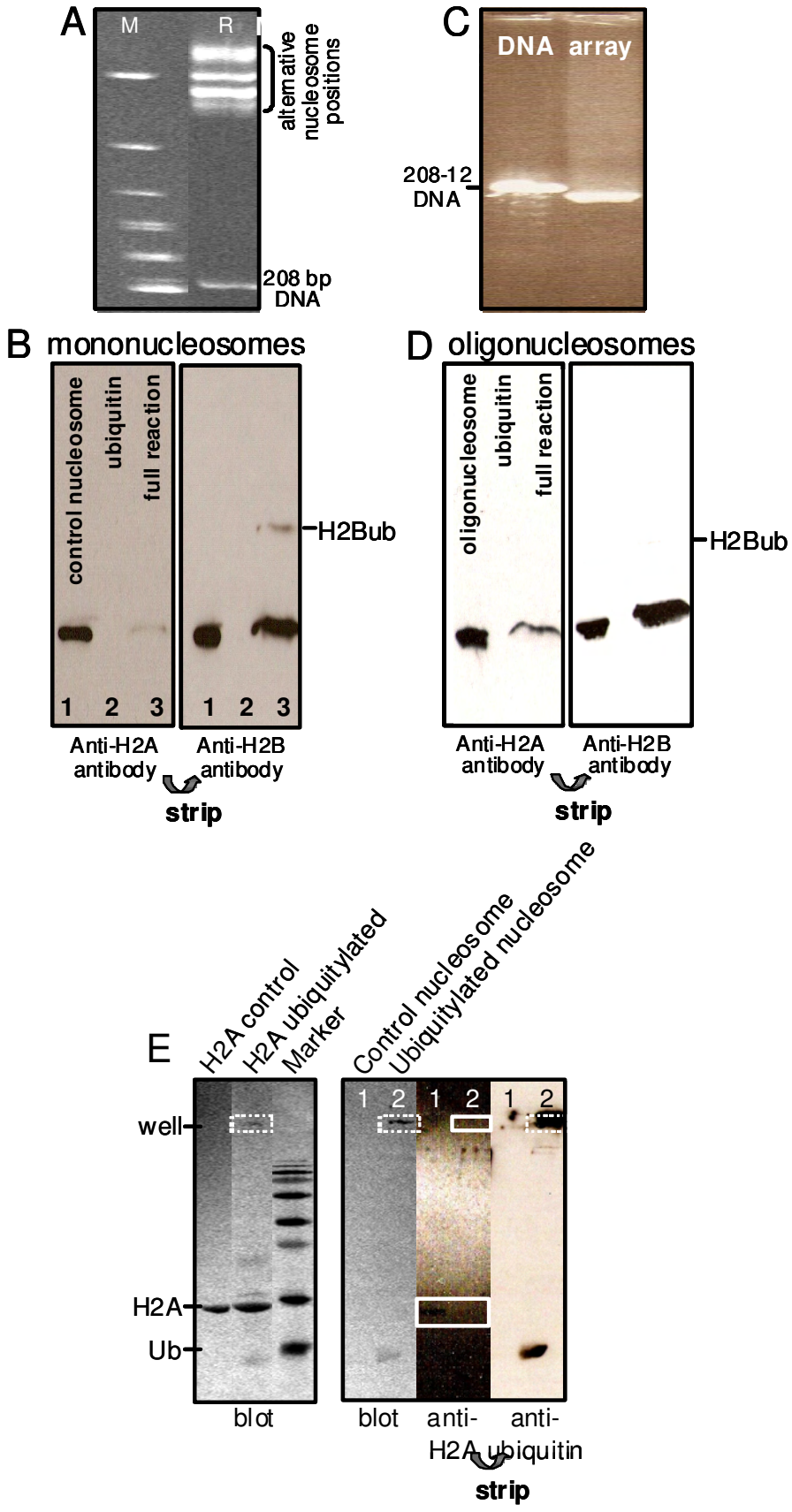

Figure 2
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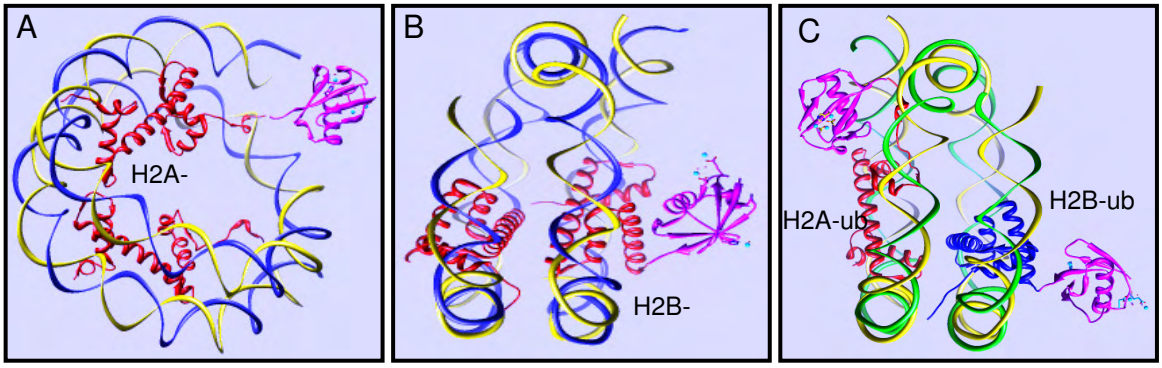

Figure 3
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